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REACTIVE OXYGEN SPECIES IN HUMAN SEMEN IN RELATION TO LEUKOCYTE CONTAMINATION

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Aims: Excessive production of reactive oxygen species (ROS) in semen has been linked to male infertility. Main sources of ROS in male genital tract are immature and/or damaged spermatozoa and a subpopulation of leukocytes known as polymorphonuclear neutrophils (PMN).

Methods: Study group included male partners of infertile couples, 67 normospermic males (group B) and 98 males with sperm abnormalities in one or more parameters (group C), 36 fertile volunteers (group A) served as controls. Sperm parameters were determined according to WHO guidelines. The ROS production was measured by chemiluminiscence in sperm suspension in phosphate buffered saline.

Results: All fertile volunteers in the control group had seminal PMN concentrations below $0.5x10^6$/ml. Therefore study subjects, 67 normospermic and 98 men with sperm abnormalities, were further subdivided into two subgroups of PMN concentrations: (1) < $0.5x10^6$/ml and (2) 0.5 to $1.0x10^6$/ml. The ROS production in individuals varied greatly from $1.0x10^2$ to $1.7 x10^7$ RLU/min per $20x10^6$ spermatozoa. The ROS production in both subgroups of normospermic men and the subgroup (1) of men with sperm abnormalities was not different from the ROS production in fertile controls. The ROS production in the subgroup (2) with sperm abnormalities was significantly higher than in controls ($P = 0.00004$).

Conclusions: Our findings suggest that the contribution of PMN to the ROS production in semen is negligible only up to a concentration of $0.5x10^6$/ml. This suggests that the current WHO Guidelines threshold of $1.0x10^6$ PMN per ml of semen is too high and might be re-evaluated.

INTRODUCTION

Semen analysis is an essential component of the investigation of male fertility. The extent of examination and the techniques used (manual, semi-automatic or automatic computer-assisted semen analysis) varies. An inherent part of such analyses is the Endtz test which detects a subpopulation of white blood cells (WBC) known as polymorphonuclear neutrophils (PMN) (ref.[1]). According to WHO Guidelines[2] the cut-off limit for the presence of PMN in human semen is $1.0 x10^6$/ml. These WBC, an integral part of defence system against infection, can produce large quantities of reactive oxygen species (ROS) during an oxidative burst[3]. When activated, PMN produce ROS significantly more than other known sources such as immature or morphologically abnormal spermatozoa[4].

ROS have contrasting roles in male fertility. In physiologically small amounts, ROS are necessary for sperm capacitation, sperm hyperactivation, acrosome reaction and sperm-oocyte fusion[5]. In large amounts, they are toxic to sperm. The lack of cytoplasmic defences renders spermatozoa more susceptible to oxidative stress[6, 7]. Moreover, spermatozoa are particularly sensitive to oxidative damage by lipid peroxidation due to the high content of polyunsaturated fatty acids, mainly docosahexaenoic acid[8] in their membranes. ROS also diminish intracellular ATP, which in turn leads to a reduction in axonemal protein phosphorylation and to sperm immobilization[4]. The latest reports stress the association of excessive ROS generation with DNA strand breaks[9, 10] and chromosome deletions in spermatozoa[11]. These changes may not be detected by routine semen analysis although they can cause reproductive failure.

Under physiological conditions ROS levels in ejaculates is well-controlled by enzymatic and non-enzymatic antioxidant systems in seminal plasma. Pathological conditions, in contrast, can lead to an imbalance between ROS production and the antioxidant defense system and thus create oxidative stress (OS) which is known to have a negative effect on male fertility.

The contribution of leukocytospermia to impairming the fertilizing ability of sperm was first studied in the mid-nineteen eighties[12], but since then conflicting opinions have been expressed. For instance, Ochsendorf[8] stated that only very high numbers of leukocytes, at least $>2.0x10^6$ PMN per ml, generate excessive ROS which

can not be counteracted by the antioxidant capacity of seminal plasma. Moskovtsev[13] found only weak correlation between WBC concentration in ejaculate and sperm DNA fragmentation index but a significant negative effect of WBC on the standard semen parametres. In contrast, Sharma et al[14] observed that any presence of leukocytes between 0 and $1.0x10^6$/ml was associated with oxidative stress and they were unable to determine a "safe" minimal leukocyte count. Our study aims to bring more evidence to bear on the relationship between PMN counts in human semen and total ROS production measured in sperm suspensions.

MATERIALS AND METHODS

The study population

The study was approved by the Institutional Review Board of Palacky University. All subjects gave their written consent. The study group included male partners of 185 consecutive infertile couples referred to the programme of assisted reproduction at the Department of Obstetrics and Gynaecology, Faculty of Medicine and Dentistry Palacky University in 2006 and 2007. Reference semen samples were collected from 36 fertile volunteers who had fathered a child in the previous 12 months. Serum levels of C-reactive protein (CRP) were determined as a routine marker in all subjects to exclude the presence of an acute inflammation at the time of semen collection.

Semen collection and analysis

Semen samples were collected by masturbation after a period of 3 to 5 days of sexual abstinence. After liquefaction (37° C, 30 minutes), smears of neat semen were prepared for sperm morphology assessment (Papanicolaou staining). Semen analysis was performed to measure sperm concentration and percentage of motility using WHO criteria[2]. A sample was classified as normospermic if semen volume was ≥ 2 ml, sperm concentration was ≥ 20×10^6 per ml, sperm motility was ≥ 50 %, normal sperm morphology ≥ 30 % and PMN detected by Endtz test ≤ 1×10^6 per ml of ejaculate. Samples which did not fulfil one or more of these criteria were classified as abnormal.

Determination of PMN leukocyte concentration

The presence of PMN leukocytes in semen was assessed by myeloperoxidase (Endtz) test[1]. Twenty µl of liquefied semen was mixed with 20 µl phosphate buffered saline (PBS; pH 7.0) and 40 µl of benzidine (4,4'-diaminobiphenyl, 13.6 mM, Sigma Aldrich). After mixing the sample was left for 5 minutes at room temperature. Peroxidase-positive (brown-stained) leukocytes were counted in a Bürker's chamber (Superior GmBH, Marienfeld, Germany) and concentration per ml of ejaculate was calculated.

ROS detection by chemiluminescent assay

Aliquots of liquefied semen were centrifuged at 300×g for 7 min. The sperm pellets were washed with PBS and resuspended in 1 ml of PBS and further diluted to a concentration of 20 million spermatozoa per ml. In the case of oligospermia (sperm concentration below 20×10^6 per ml) the luminescent signal was appropriately multiplied to correspond to 20×10^6 per ml. ROS production was estimated within 30 min after liquefaction by chemiluminescence following the addition of 10 µl of 5 mM luminol (5-amino-2,3-dihydro-1,4 phthalazinedione, Sigma Aldrich) in dimethyl sulfoxide (DMSO, Sigma Aldrich) to 400 µl of sperm suspension. Blank tube containing 400 µl of PBS buffer and 5 µl of Luminol was used as assay control. Light emission was measured for 15 min using a DCR-1 luminometer (Digene Diagnostics, Inc.). The results were expressed as relative light units RLU/20×10^6 sperm/15 min. The method was described in detail by Novotny et al[15].

Statistical analysis

STATISTICA Cz data analysis software (version 6; StatSoft Inc, 2001) was used for statistical calculations. Summary statistics are presented as mean and standard deviation (±SD) for Gaussian distributed variables and medians for non-Gaussian ones. Due to the extremely large range of absolute values of ROS production statistical comparisons of ROS levels among groups was performed after logarithmic transformation of RLU/min. Kruskal-Wallis ANOVA and non-parametric Mann-Whitney U test were utilized to compare ROS level between groups. The Student t test was used for comparison of sperm parameters. All hypothesis testing was two-tailed and $p<0.05$ was considered statistically significant.

RESULTS

Of the 185 consecutive patients referred with their partners for assisted conception 20 (10.8 %) were excluded from further evaluation as their ejaculate did not contain enough sperm (at least $5x10^6$ spermatozoa/ml) to allow a reliable ROS measurement. Of the remaining 165 patients 67 (40.6 %) were normospermic (group B) and 98 (59.4 %) had abnormalities in one or more sperm parameter (group C). There was no evidence of significant difference in mean age between healthy volunteers (group A), group B patients and group C patients (30.5, 32.4, and 33 years; P = 0.148). Serum levels of CRP determined at the time of semen collection confirmed the absence of inflammation in all males and none of them reported any acute health problems.

Sperm parameters

There was no significant difference in the volume of ejaculates between groups A, B and C (Table 1). Groups A and B had similar sperm concentration, total sperm count and percentage normal morphology, however sperm motility was significantly higher in group A than group B. Group C patients has significantly lower sperm count, percentage motility and normal morphology than groups A or B (Table 1).

Table1. Spermiogram parameters (mean ± SD) in healthy volunteers (A) and men of infertile couples (B and C). Mann-Whitney U test was utilized to compare groups.

	group A (n=36) mean ± SD	group B (n=67) mean ± SD	group C (n=98) mean ± SD	A: B p	A: C p	B:C p
ejaculate volume (ml)	3.1 ± 1.3	3.3 ± 1.2	3.0 ± 1.5	0.352	0.912	0.213
sperm concentration (10^6/ml)	69.6 ± 35.6	66.7 ± 28.4	39.8 ± 27.6	0.942	0.00003	0.00004
total sperm count (x10^6)	198 ± 96	213 ± 122	115 ± 95	0.857	0.00004	0.00002
motility (%)	50 ± 8	53 ± 5	38 ± 13	0.030	0.00002	0.00001
normal morphology (%)	35.9 ± 9.4	38.0 ± 6.8	21.5 ± 8.5	0.230	0.00001	0.00001

Table 2. ROS production in medians and 90th centiles of log ROS and corresponding ROS production in RLU/min.

ROS production	group A (n = 36)	subgroup B1 (n = 59)	subgroup C1 (n = 84)	A : B1 p	A : C1 p	B1 : C1 p
median log ROS	3.015	2.698	3.317	0.178	0.439	0.026
approx. in RLU/min	1 000	500	2 100			
90th centile log ROS	4.367	4.357	4.731			
approx. in RLU/min	23 200	22 800	53 800			
		subgroup B2 (n = 8)	subgroup C2 (n = 10)	A : B2 p	A : C2 p	B2 : C2 p
median log ROS	3.015	3.737	5.185	0.394	0.00004	0.008
approx. in RLU/min	1 000	5 600	153 000			
90th centile log ROS	4.367	4.904	5.866			
approx. in RLU/min	23 200	80 200	735 000			
		B1 + B2 (n=67)	C1+C2 (n=94)	B1 : B2 p	C1 : C2 p	B : C p
median log ROS		2.782	3.424	0.158	0.0002	0.0004
approx. in RLU/min		605	2 656			
90th centile log ROS		4.406	5.155			
approx. in RLU/min		25 725	143 326			

Endtz test

Seminal PMN concentration was lower than 0.5x10^6/ml in all fertile volunteers. Only 4 males from group C had leukocytospermia as defined by WHO and were excluded from statistical analysis. All non-leukocytospermic subjects were further subdivided on the basis of seminal PMN concentration into two subgroups: (1) < 0.5x10^6/ml and (2) 0.5x10^6/ml to 1x 10^6 PMN/ml.

Among group B patients, 59 were in subgroup 1 (B1) and 8 in subgroup 2 (B2). Among the C group patients, 84 men had a PMN concentration < 0.5x10^6/ml (C1) and 10 men had PMN concentration > 0.5x10^6/ml (C2).

ROS production

The results of the luminol chemiluminescence assay varied greatly from 100 to 115 000 RLU/min generated by 20×10^6 spermatozoa in our healthy fertile volunteers, from 100 to 354 000 RLU/min in normospermic study subjects and from 100 up to 16 million in men with sperm abnormalities. Due to this extremely large range of values, logarithms of ROS production in RLU/min were used for non-parametric statistical evaluation (Table 2). When the three groups (A, B, C) were compared, group C patients had significantly higher median log ROS level than group A or B (P= 0.0002, P= 0.0004, respectively), whereas groups A and B had similar median log ROS level (P= 0.158). Within group comparison based on PMN count

cut-off of $0.5x10^6$/ml revealed that C2 subgroup had significantly higher median log ROS level than subgroup C1 (P=0.0002). However, although the median ROS production in the normospermic subgroup B2 (n = 8) was over ten times higher than subgroup B1, the difference did not reach statistical significance. Between subgroup comparison revealed that B1 subgroup had significantly lower median log ROS level than C1 subgroup (P=0.026). Similarly, B2 subgroup had significantly lower median log ROS level than C2 subgroup (P=0.008). There were no significant differences in median ROS levels between group A and subgroups B1 or subgroup C1.

DISCUSSION

In this study we compared healthy fertile volunteers with the male partners of couples presenting with infertility, to evaluate semen leukocyte count and ROS level. Patients with normal spermiogram (group B) had significantly lower ROS level than patients with abnormal spermiogram (group C). All fertile volunteers had PMN count < $0.5x10^6$/ml. Within group B and group C, the ROS level was lower in those with PMN count < $0.5x10^6$/ml compared to those with PMN count $0.5x10^6$/ml to $1x10^6$/ml. However the difference reached statistical significance only in group C.

Inspite of the known negative effects of oxidative stress on male fertility and the reported beneficial clinical effects of anti-inflammatory and antioxidant treatments in cases of male infertility[16-19], the quantitative aspects of free radical production in human semen are still poorly understood. The reasons for this are several. First, the direct measurement of ROS production is almost impossible due to their extremely short half-life. Secondly, modern methods such as Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR) spectroscopy[20] require sophisticated instrumentation, are time consuming and hence not suitable for clinical practice. Thirdly, though the relatively simple indirect methods based on ROS-triggered luminol or lucigenin chemiluminescence have been used for measurement in human sperm[15, 21, 22] for about 20 years, the absolute data from different settings, referred to alternatively as photons, counts or RLU per minute, are difficult to compare because counting efficiency is unique for a given luminometer. Finally, measurement of ROS in washed sperm suspension is performed in an artificial environment with depleted antioxidants. A promising approach to circumvent this problem may be to assess the actual oxidant/antioxidant balance by measurement of ROS production in neat semen[23].

Excessive ROS in the male genital tract may be generated by two cell types: immature and/or pathological spermatozoa and activated PMN and/or macrophages. Opinions continue to differ about the relative contribution of individual radicals from these two sources to the total ROS production in semen, although logically, the immature or pathologic spermatozoa in males with sperm abnormalities would be expected to make a greater contribution than in normospermic males[22]. This possibility was actually reflected in our finding of significantly higher ROS levels in subgroup C1 compared to B1 (P=0.026). The highest increase in ROS production was found in 10 males from subgroup C2 with PMN concentrations between $0.5x10^6$/ml and $1.0x10^6$/ml (P= 0.00004) compared to fertile volunteers. Our findings therefore provide additional evidence of the importance of leukocyte infiltration in excessive ROS production in human semen.

Spermatozoa produce mainly superoxide which reacts with nitric oxide to form peroxynitrite, a putative mediator of oxidative cellular injury. But activated PMN and macrophages produce a more persistent molecule, H_2O_2, which can penetrate plasma membranes[13], and which may easily form a very strong oxidant hydroxyl-radical ($\cdot OH^-$) in Fenton reaction[3]. In 1999, Ochsendorf[8] suggested that only very high leukocyte contamination of semen (over $2.0x10^6$ /ml) produces ROS which cannot be efficiently scavenged by the antioxidant capacity of seminal plasma. In contrast to this report, we found no semen with more than $2.0x10^6$ PMN/ml and yet we observed some extremely high levels of ROS production. Recently, several authors have suggested that even much lower concentrations of PMN can produce detrimental amounts of ROS[24]. Sharma et al[25] observed that any presence of PMN between 0 and $1.0x10^6$/ml was associated with oxidative stress and were unable to determine a "safe" minimal leukocyte count. Henkel et al[26] analyzed ROS production in 63 non-leukocytospermic patients and claimed that the "intrinsic" ROS production (percentage of spermatozoa producing ROS) calculated using dihydroethidine as a probe in a microslide smear of sperm suspension in PBS is significantly higher in patients with more than as little as $0.1x10^6$ leukocytes per ml of ejaculate.

Using a combination of EPR, chemiluminiscence and nitroblue tetrazolium dye reduction, Armstrong et al[20] also found that ROS-producing activity of spermatozoa may be different and significantly lower than that of leukocytes. The mechanism of ROS generation in human sperm depends upon a novel NADPH-oxidase (NOX5) resembling the multicomponent NADPH-oxidase of leukocytes, but having a significantly lower activity. The pro-oxidative potential of leukocytes in semen may be further enhanced by bacterial products and cytokines.

Kovalski et al[19] warned that H_2O_2 and $\cdot OH$ produced by concentrations of PMN in semen as low as $0.6x10^6$/ml can be hazardous for spermatozoa, as seminal plasma may not always provide sufficient antioxidant protection. Recent measurements using flow-cytometry and monoclonal antibodies may be helpful in the identification of WBC subpopulations in human semen[27]. Based on their results the authors suggested lowering the WHO threshold value for leukocytospermia to $0.2x10^6$ PMN/ml. Another study confirmed that seminal PMN below $1.0x10^6$ may be associated with increased ROS generation[24].

The conflict of opinion concerning the correct threshold for detrimental leukocyte infiltration in human semen may be explained by the fact that within normal semen there may exist sperm subpopulations with different bio-

chemical control mechanisms enabling them to defend themselves against leukocyte-derived ROS[28]. These authors also stress the need for a more detailed evaluation of leukocyte contamination of seminal fluid in order to better understand their role in oxidative stress.

CONCLUSIONS

In conclusion, our finding of increased ROS production in men from infertile couples with leukocyte contamination exceeding the level of $0.5x10^6$ PMN/ml in ejaculate are consistent with some of these reports[19, 24]. In the light of the acknowledged negative effects of oxidative stress on fertility potential of spermatozoa especially in assisted reproduction procedures we also suggest that the current WHO Guidelines threshold for normospermia of $1.0x10^6$ PMN/ml is too high and should be re-evaluated.

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